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Signaling through CD44 Is Mediated by Tyrosine Kinases

ASSOCIATION WITH p56^{lck} IN T LYMPHOCYTES*

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Evidence from a large body of studies indicates that CD44 is involved in a number of important biological processes, including lymphocyte activation and homing, hematopoiesis, and tumor progression and metastasis. A proper understanding of the role of CD44 in these processes has been severely hampered by a lack of insight into the mode in which CD44 communicates with intracellular signal transduction pathways. In this report, we have addressed this aspect of CD44 functioning by studying CD44 signaling in T lymphocytes. We show that ligation of CD44 by monoclonal antibodies (mAbs) transduces signals to T cells which lead to tyrosine phosphorylation of ZAP-70 and other intracellular proteins. *In vitro* kinase assays demonstrate that cross-linking of CD44 induces an increase in the intrinsic activity of p56^{lck}. Furthermore, immunoprecipitations show that CD44 is physically associated with p56^{lck}. Our findings suggest that tyrosine kinases, particularly p56^{lck}, play a central role in CD44 mediated signaling.

CD44 is a broadly distributed family of cell surface glycoproteins involved in cell-cell and cell matrix adhesion (1–7). Although the exact spectrum of functions of CD44 is presently unknown, members of the CD44 family have been implicated in a number of important biological processes, including lymphocyte functioning, hematopoiesis, and tumor progression and metastasis (1, 6, 8–15). The CD44 gene consists of 20 exons (16). Due to alternative RNA splicing which involves at least 10 exons encoding domains of the extracellular portion of the CD44 molecule, a large number of CD44 isoforms are generated. In addition to variable exon usage, variations in glycosylation contribute to the structural and functional diversity of CD44 (1).

A widely expressed CD44 isoform is the "standard" or "hematopoietic" CD44 (CD44s) molecule (1, 4, 10). On hematopoietic cells and lymphocytes this 85–95-kDa molecule is the principle CD44 isoform (4, 10, 17–19). Larger CD44 variants that contain different combinations of alternatively spliced exons are preferentially expressed on epithelial cells (17–19), but they can also be found on activated lymphocytes (10, 20) and high grade malignant lymphomas (10). During lymphocyte ontogeny and activation the expression of CD44 is strictly regulated, suggesting an important functional role for CD44 (1, 8, 21). Indeed, CD44 has been reported to be involved in a variety of lymphocyte functions including lymphopoiesis (11), lymphocyte homing (6), and lymphocyte activation (22–26). In TCR[1]·CD3- and CD2-mediated T cell activation, CD44 can function as an important costimulatory molecule leading to enhanced proliferation and cytokine release (22–25). Furthermore, engagement of CD44 can lead to activation of the integrin LFA-1 (CD11a/18) on the cell surface of T lymphocytes resulting in enhanced adhesiveness (26). Taken together, these data suggest that CD44 functions as an important signaling molecule in immune interactions; however, the signal transduction pathways involved in this CD44-mediated signaling are unknown.

Phosphorylation of proteins on tyrosine residues through protein tyrosine kinases is a key event in the regulation of cell growth and differentiation (27). In T lymphocytes, they play a pivotal role in antigen-specific activation and proliferation (28). In the present study, we have therefore explored the possible role of protein tyrosine kinases in CD44-mediated signaling. The results show that triggering of CD44 transduces signals across the plasma membrane that lead to tyrosine phosphorylation of ZAP-70 and other intracellular proteins. Furthermore, we demonstrate a physical association between the protein tyrosine kinase p56^{lck} and the CD44 molecule, suggesting its importance in the CD44 signaling pathway.

MATERIALS AND METHODS

Cells and Antibodies—Peripheral blood mononuclear cells from buffy coat preparations were isolated by Ficoll-Isopaque density gradient centrifugation. Purified T lymphocytes were either prepared by rosetting the cells with sheep erythrocytes or by incubation on ice with a mixture containing saturating concentration of mAbs against CD14, CD11b, and CD20 (Dako, Glostrup, Denmark) followed by immunomagnetic depletion of the antibody-coated cells by two successive round of incubations with goat anti-mouse Ig (GαM)-conjugated magnetic beads (Dynal, Oslo, Norway). The T cell-enriched preparations were >98% CD3$^+$. COS7 cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% glutamine, penicillin, and streptomycin.

For triggering or precipitation of CD44, the mAbs Hermes-3 (29), J173 (Immunotech S.A., Marseille, France), and NKI-P1 (5) directed against epitopes on the standard part of CD44 were used. Biotinylated Leu-4 (Becton Dickinson, Mountain View, CA) was used for triggering of CD3. Polyclonal antibodies against p56^{lck} (30) alone or together with swine anti-rabbit Ig (SαR) were used for immunoprecipitation of the p56^{lck}. Polyclonal antibodies against ZAP-70 (kindly provided by Dr. Arthur Weiss, University of California, San Francisco, CA) were used for immunoprecipitation and immunoblotting of ZAP-70. GαM Ig

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[1] The abbreviations used are: TCR, T cell receptor; mAb, monoclonal antibody; HRP, horseradish peroxidase; PAGE. polyacrylamide gel electrophoresis; PY, phosphotyrosine; GαM, goat anti-mouse Ig; T-PBL, peripheral blood T lymphocyte.

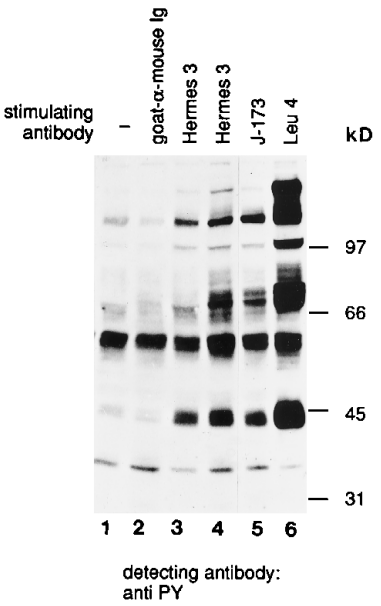

FIG. 1. **Induction of tyrosine phosphorylation by CD44 triggering in resting peripheral blood T lymphocytes (T-PBL).** Anti-PY stained Western blot of purified T cells that were triggered with anti-CD44 mAbs (Hermes-3 and J173) or anti-CD3 mAb (Leu-4) and cross-linking. At two (*lanes 2*, *3*, *5*, and *6*) or 5 min (*lane 4*) after the addition of the cross-linker, the cells were lysed in Nonidet P-40 sample buffer. 2 × 10^6 cells/lane were separated on 8% SDS-PAGE, transferred to nitrocellulose, and then analyzed by Western blot using mAb PY20-HRP followed by ECL chemiluminescence detection. T cells were incubated with *lane 1*, PBS alone; *lane 2*, cross-linker (GαM) alone; *lane 3*, Hermes-3 plus GαM; *lane 4*, Hermes-3 plus GαM; *lane 5*, J173 plus GαM; *lane 6*, biotinylated Leu-4 plus streptavidin.

(Southern Biotechnology Inc. Birmingham, AL) and Steptavidin (Sigma) were used for cross-linking of CD44 or CD3 receptors. Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine (PY) (PY20-HRP) (Affiniti, Nottingham, UK) was used for the detection of tyrosine phosphorylated proteins. mAbs against p56^{lck} (Santa Cruz Biotechnology Inc.) or Hermes-3, together with HRP-conjugated rabbit anti-mouse Ig were used for detecting the p56^{lck} or CD44 proteins on blots. Polyclonal antibody against p56^{lck} together with HRP-conjugated goat anti-rabbit Ig-HRP were also used to detect p56^{lck}. mAb against CD27, 2E4 (kindly provided by Dr. Rene van Lier, CLB, Amsterdam, The Netherlands) were used for immunoprecipitation of CD27.

T Cell Triggering and Tyrosine Phosphorylation—Purified T cells (2.106) were washed twice in phosphate-buffered saline and subsequently incubated in the presence or absence of 10 μg/ml Hermes-3, J173, or biotinylated Leu-4 for 5 min at 37 °C. After washing in phosphate-buffered saline, the cells were suspended in 50 μg/ml GαM Ig or streptavidin for 2 min. The reaction was stopped by the addition of ice-cold 2 × lysis buffer to a final concentration of 50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40 (Fluka, Buchs, Switzerland), 10 μg/ml leupeptin (Sigma), 10 μg/ml aprotinin (Sigma), 1 mM sodium orthovandate (Sigma), 5 mM NaF, and 2 mM EDTA. The insoluble nuclear material was removed by centrifugation at 14,000 rpm at 4 °C for 20 min. Supernatants were diluted in Laemmli sample buffer (to 1 × sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, and 0.001% bromphenol blue) and boiled for 5 min. Proteins were electrophoresed on 8% SDS-PAGE, transferred to nitrocellulose sheets (Schleicher & Schuell, Dasel, Germany), blocked with 2% blot qualified bovine serum albumin (Promega, Madison, WI) in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), and then incubated with PY20-HRP (0.2 μg/ml). Reaction was revealed with an enhanced chemiluminescence system (ECL; Amersham, Paris, France).

Immunoprecipitation—For immunoprecipitation, cell lysates of 2 × 10^7 stimulated or nonstimulated cells were prepared as described above and proceeded to preclearing for 45 min with Sepharose beads coupled to normal mouse serum, to normal rabbit serum, or to protein A (Pharmacia Biotech Inc.). Immune complexes were collected with Sepharose beads directly coupled to Hermes-3 or NKI-P1. In addition, immune complexes were collected with either Sepharose beads directly coupled to swine anti-rabbit Ig and then loaded with polyclonal antibody against p56^{lck} tyrosine kinase or with protein A-Sepharose beads coupled to polyclonal antibody against p56^{lck}. The immune precipitates were washed five times with 1 × lysis buffer. The proteins were then eluted and dissolved by boiling for 5 min in Laemmli sample buffer and proceeded to Western blot analysis as described above, except that the blots were blocked with 5% nonfatty dry milk in TBST in the case of incubation of the blots with Hermes-3, with anti-p56^{lck}, or with anti-ZAP-70. Antibodies bound to CD44 or to the phosphotyrosine on the blots were detected by the addition rabbit anti-mouse-HRP, whereas antibodies bound to p56^{lck} or ZAP-70 on the blots were detected by the addition of goat anti-rabbit-HRP, and proceeded to ECL.

Immune Complex Kinase Assays—The immune complexes from 2 × 10^7 stimulated or nonstimulated T cells were washed three times with 1 × lysis buffer followed by washing twice with kinase buffer (20 mM Tris, pH 7.0, 5 mM MgCl, 5 mM MnCl), suspended in 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP, and incubated for 10 min at room temperature. The reaction was stopped by addition of 1 ml of kinase buffer and the immune complex was washed twice with the same buffer. After addition of 1 × Laemmli sample buffer and boiling for 5 min, the proteins were separated on 8 or 10% SDS-PAGE, the gel was dried for 3 h, and the drying gel was autoradiographed at −80 °C overnight.

Transfections—COS7 cells were transfected with plasmids DNA containing CD44s or p56^{lck} cDNA inserts using DEAE-dextran. Briefly, COS7 cells were plated at a density of 2 × 10^6 cells/100-mm dish and transfected with 5 μg of plasmids DNA. Transfected cells were harvested after 48 h, analyzed for the expression of CD44 and p56^{lck}, and subjected to immunoprecipitation.

RESULTS AND DISCUSSION

Triggering of CD44 on resting peripheral blood T cells with either of two anti-CD44 mAbs that recognize epitopes on CD44s (Hermes-3 and J173) induced a rapid and important increase of tyrosine phosphorylation of several intracellular proteins, including substrates with a molecular mass of approximately 140, 125, 100, 70, 55, and 45 kDa (Fig. 1). This indicated that CD44 might be physically associated with intracellular kinase(s). To assess this possibility, the kinase activity of CD44 immunoprecipitates of Nonidet P-40-treated T cell lysates was studied by means of the *in vitro* immune complex kinase assay. As is shown in Fig. 2, the anti-CD44 precipitates yielded a major phosphorylated protein, migrating at approximately 55–60 kDa.

To identify the kinase(s) associated with CD44, a series of immunoprecipitations using antibodies against CD44 and Src-family tyrosine kinases were performed. Immunoprecipitation with anti-CD44 co-precipitated a 55–60-kDa protein reacting with mAb PY20 against PY (not shown). Further studies showed that this protein reacted also with monoclonal and polyclonal antibodies against p56^{lck} (Fig. 3). Hence, precipitation of CD44 leads to co-precipitation of p56^{lck}, strongly suggesting a physical association between these two molecules. To ascertain this finding, reverse precipitations were performed using antibodies against p56^{lck}. These studies demonstrated that precipitation of p56^{lck} leads to co-precipitation of CD44 (Fig. 4). Furthermore, experiments using COS cells that had been co-transfected with CD44 and p56^{lck} cDNAs also confirmed the association between CD44 and p56^{lck}; immunoprecipitation of CD44 from these cells co-precipitated p56^{lck} (Fig. 5). Hence, CD44 is physically associated with p56^{lck} a finding which suggests that p56^{lck} might be functionally involved in the signal transduction via CD44.

To substantiate the notion of a functional association between CD44 and p56^{lck}, we next measured the effect of CD44 triggering on the intrinsic kinase activity of p56^{lck} and on the phosphorylation state of ZAP-70, a substrate of p56^{lck} (31). As is shown in Fig. 6, cross-linking of CD44 induced a time-dependent increase in the tyrosine kinase activity of p56^{lck}, as measured by the *in vitro* kinase assay, with an optimum at 2–5 min after cross-linking. Only in the precipitates of p56^{lck}, obtained after cross-linking of CD44, prolonged exposure of the

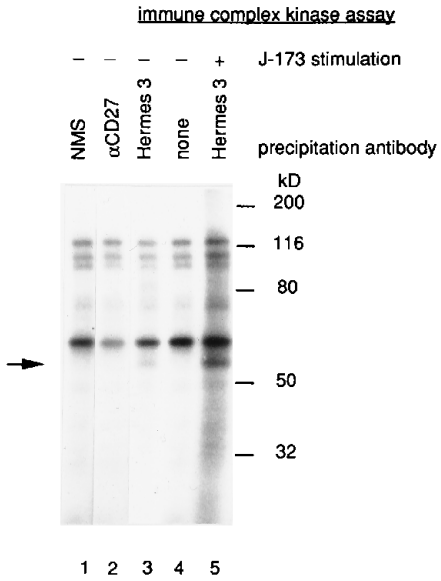

FIG. 2. **CD44 is associated with kinase activity.** Kinase activity of CD44 immunoprecipitates from T-PBL (with mAb Hermes-3) was assessed by means of the immune complex kinase assay as detailed in materials and methods. *lane 1*, Control precipitate (beads coupled to normal mouse serum (*NMS*)); *lane 2*, precipitate of CD27; *lane 3*, precipitate of CD44 from unstimulated T-PBL; *lane 4*, control precipitate (uncoated beads); *lane 5*, precipitate of CD44 from CD44 (with mAb J173)-triggered T-PBL. The *arrow* points at a 55–60-kDa ^{32}P-labeled protein detected only in the immunoprecipitates of CD44.

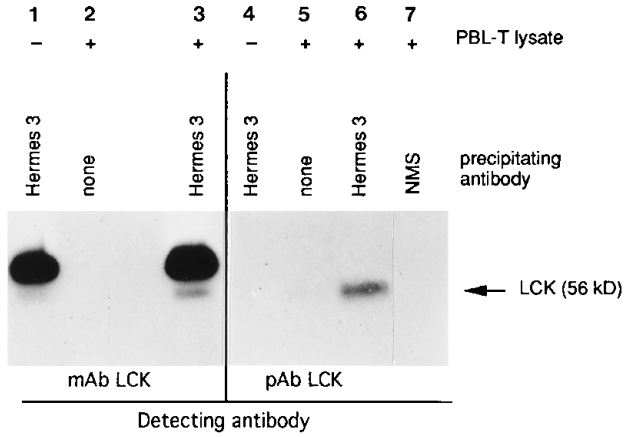

FIG. 3. **Co-precipitation of p56^{lck} with CD44.** Western blot analysis of CD44 immunoprecipitates from T-PBL stained either with mAb (*lanes 1*, *2*, and *3*) or polyclonal antibody (*lanes 4*, *5*, *6*, and *7*) against p56^{lck}. *Lanes 1* and *4*, antibody controls; *lanes 2* and *5*, control precipitates (unlabeled beads); *lanes 3* and *6*, immunoprecipitates of CD44; *lane 7*, control precipitate (beads coupled to normal mouse serum (*NMS*)). The *arrow* points at the p56^{lck} band in the immunoprecipitates of CD44. The strong band of approximately 60 kDa in *lanes 1* and *3* represents the Ig heavy chain of Hermes-3.

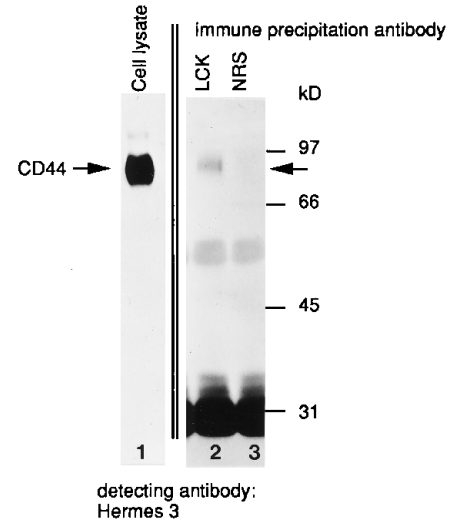

FIG. 4. **Co-precipitation of CD44 with p56^{lck}.** Western blot analysis, of *lane 1*, the total cell lysate of T-PBL; *lane 2*, p56^{lck}; and *lane 3*, control (normal rabbit serum (*NRS*)) immunoprecipitates from T-PBL, using anti-CD44 mAb (Hermes-3). The *arrow* points at the CD44 molecule (about 90 kDa). The bands at 55 kDa and 28–35 kDa represent the Ig chains of the Swine antibodies.

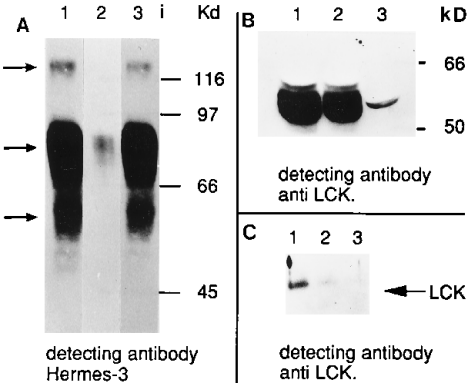

FIG. 5. **Co-precipitations of P56^{lck} with CD44 from COS cells co-transfected with CD44 and p56^{lck} cDNAs.** *A*, *B*, and *C*, COS7 cells were transfected with plasmid DNA containing CD44 and p56^{lck} inserts (*lane 1*), p56^{lck} inserts alone (*lane 2*), and CD44 inserts alone (*lane 3*). *A*, anti-CD44 (Hermes-3) stained Western blot of the lysates of COS-7; CD44 is detected in *lanes 1* and *3*. The *arrows* point at different forms of CD44. *B*, anti-p56^{lck}-stained Western blot of the lysates of COS7; p56^{lck} is detected in *lanes 1* and *2*. *C*, anti-p56^{lck}-stained Western blot of the CD44 immunoprecipitate from COS7. The *arrow* points at the p56^{lck} molecule in the immunoprecipitate.

gel revealed the presence of additional phosphorylated proteins (data not shown). This finding is in agreement with the fact that several intracellular proteins complex with p56^{lck} upon its activation. ZAP-70, a tyrosine kinase that becomes tyrosine-phosphorylated in *lck*-dependent manner, was found to become tyrosine-phosphorylated after cross-linking of CD44 (Fig. 7). Together, these findings establish that CD44 is functionally linked to p56^{lck}.

p56^{lck} plays a key role in thymocyte development and TCR·CD3-mediated signaling (32, 33). In a mutant clone of the Jurkat T leukemia line deficient in *lck*, signaling through the TCR·CD3 complex was severely defective; it was restored upon reconstitution with wild-type *lck* (33). Furthermore, mice lacking functional p56^{lck} show a block in early thymocyte development with a dramatic reduction of the double positive (CD4$^+$CD8$^+$) thymocyte population and absence of mature single (CD4$^+$ or CD8$^+$) positive thymocytes (34). Of the molecules involved in antigen-specific recognition via the TCR·CD3 complex, CD4 and CD8 are associated with p56^{lck} (35). In addition, several other receptors on the T cell membrane including CD2, IL-2R, CD5, and CD50 have more recently also been shown to be associated with p56^{lck} (36–39). Although *lck* plays a key role in TCR·CD3-mediated T cell activation and *lck* can be recruited into the TCR complex via CD4 and CD8, recent studies have indicated that binding of *lck* to CD4 is not required for the strongly potentiating effect of CD4 engagement on TCR·CD3-mediated T cell activation (40). Presumably, *lck*, being essential for TCR-mediated signaling, may also be recruited into the TCR·CD3 complex from other sources than CD4 (or CD8). *lck* bound to CD44 might be one of these sources and CD44 might directly or indirectly interact with components of the TCR·CD3

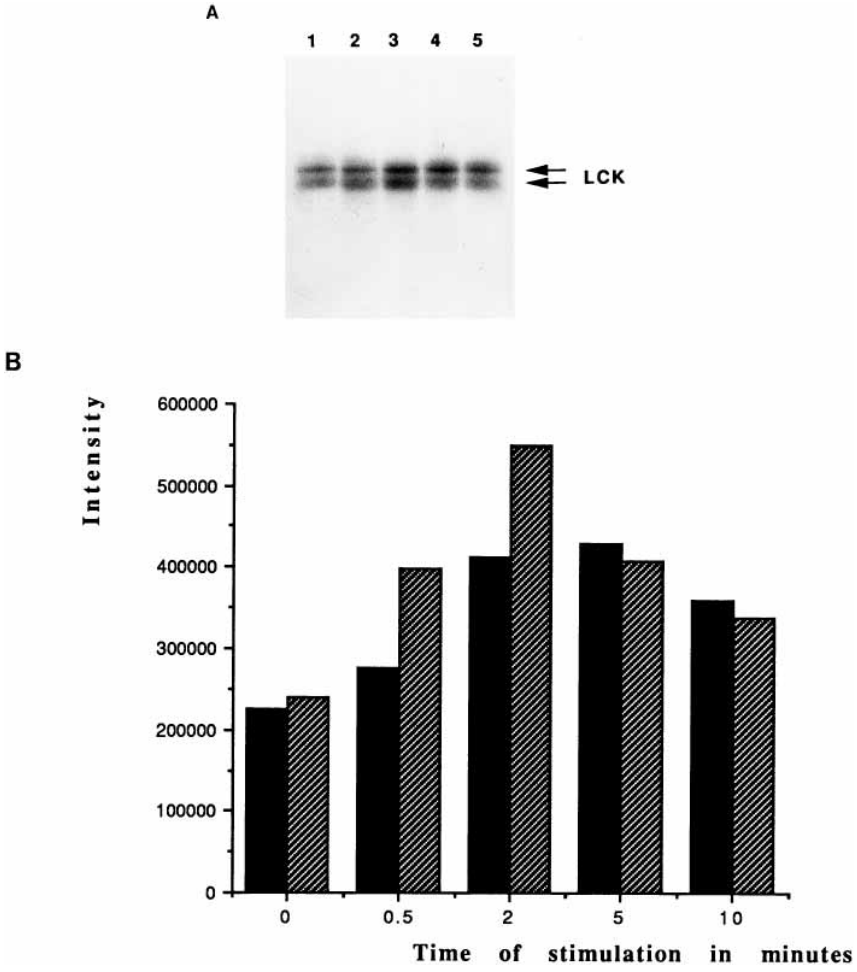

FIG. 6. **Cross-linking of CD44 induces an increase in the intrinsic activity of p56^{lck}.** The immunoprecipitates of p56^{lck} were collected by using protein A-Sepharose beads coupled to polyclonal antibodies against p56^{lck} and subjected to *in vitro* kinase assay. *A*, *lane 1*, immunoprecipitate of p56^{lck} from unstimulated T-PBL; *lanes 2–5*, the immunoprecipitates of p56^{lck} from CD44-stimulated T-PBL for 0.5 (*lane 2*), 2 (*lane 3*), 5 (*lane 4*), and 10 (*lane 5*) min. *B*, a diagrammatic representation of the intensity of the p56^{lck} bands before and after cross-linking of CD44. The *black column* represents the upper band of p56^{lck}, whereas the *striped column* represents the lower band. The optimum kinase activity (approximately 2.5-fold increase in the tyrosine kinase activity of p56^{lck}) was detected after CD44-triggering for 2–5 min.

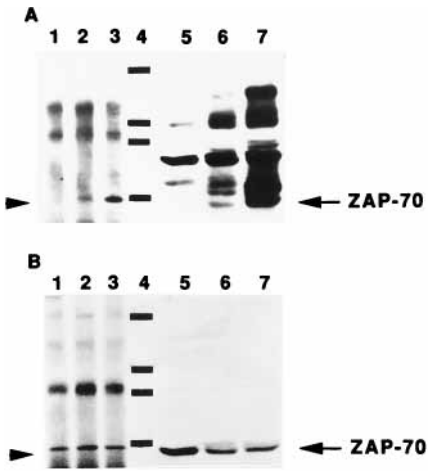

FIG. 7. **Cross-linking of CD44 induces tyrosine phosphorylation of ZAP-70.** *A*, immunoprecipitates of ZAP-70 were collected using protein A-Sepharose beads coupled with anti-ZAP-70. *Lane 1*, ZAP-70 immunoprecipitate from unstimulated T-PBL; *lane 2*, ZAP-70 immunoprecipitate from CD44-triggered cells for 3 min; *lane 3*, ZAP-70 immunoprecipitate from CD3-triggered cells; *lane 4*, molecular mass marker (from top to bottom 200, 116, 97 and 66 kDa); *lane 5*, total cell lysate of unstimulated cells; *lane 6*, total cell lysate of CD44-triggered cells; *lane 7*, total cell lysate of CD3-triggered cells. The filter was subjected to immunoblotting using αPY antibodies and rabbit anti-mouse-HRP. The *arrows* point to the tyrosine-phosphorylated ZAP-70 detected after cross-linking of CD44 and was highly phosphorylated after cross-linking of CD3. *B*, the same filter was stripped and then incubated with antibodies against αZAP-70 antibodies and goat anti-rabbit-HRP. The *arrowheads* point to ZAP-70 where the amounts of ZAP-70 precipitated are equal in *lanes 1*, *2*, and *3*, and the presence of ZAP-70 is detected in the cell lysates.

complex. Since CD44 is also abundantly expressed on hematopoietic stem cells, prothymocytes and early (CD4$^-$, CD8$^-$, and CD25$^-$) thymocytes it will be of interest to determine whether CD44 on these cells is associated with p56^{lck}. If so, CD44-*lck*-mediated signaling, triggered by hitherto undefined CD44 ligands in the bone marrow and/or thymus might play an important role in early lymphocyte development.

Our observation that CD44 in T lymphocytes is associated with p56^{lck} raises the important question how CD44 interacts with *lck*. In principle, it is possible that the intracytoplasmic domain of CD44 is directly involved in *lck* binding. Since CD44 contains serine phosphorylation sites, the interaction of CD44 with *lck* could be regulated through CD44 serine phosphorylation, in a way similar to that described for the CD4 molecule. In the CD4 molecule, the serines are, however, placed next to the p56^{lck} recognition site which contains a the characteristic cysteine motive CXCP (41) that is not present in CD44. Hence, CD44 either uses another unknown *lck*-binding motif or is indirectly associated with *lck* via a multimolecular complex.

Whether various CD44 splice variants show differential association with *lck* and with other components of the cell's signal transduction system will be another intriguing question to be answered. On resting T lymphocytes, as used in our present study, CD44 isoforms other than the standard "hematopoietic" form of CD44 are virtually absent. However, activation of T lymphocytes leads to a transient expression of several CD44 splice variants (10, 20). Although the cytoplasmic tail of these variants is identical to that of CD44s, the variations in the extracellular domain might alter CD44 association with *lck* either by affecting the conformation of the putative cytoplasmic *lck* binding domain or by modulating its interactions with

molecular partners on the T cell surface. In addition to binding to specific (hitherto undefined) ligands, the CD44 splice variants might thus act by modulating signal transduction. In this way they might regulate lymphocyte activation. In other cell types including tumor cells, they might also interact with tyrosine kinases and act as regulators of cell growth, differentiation, and tumor progression. Moreover, it will be important to determine the cascade of the signaling through CD44 and also the role of ZAP-70 in this signaling pathway.

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